ELSEVIER

Degradation of the *S. frugiperda* peritrophic matrix by an inducible maize cysteine protease

S. Mohan[a], P.W.K. Ma[b], T. Pechan[c], E.R. Bassford[a], W.P. Williams[d], D.S. Luthe[a],*

[a]*Department of Biochemistry and Molecular Biology, Box 9659, Mississippi State, MS 39762, USA*
[b]*Department of Entomology, Box 9775, Mississippi State, MS 39762, USA*
[c]*Life Science and Biotechnology Institute, Box 9758, Mississippi State, MS 39762, USA*
[d]*USDA-ARS Corn Host Plant Resistance Laboratory, Box 9555 Mississippi State, MS 39762, USA*

Abstract

A unique 33-kDa cysteine protease (Mir1-CP) rapidly accumulates at the feeding site in the whorls of maize (*Zea mays* L.) lines that are resistant to herbivory by *Spodoptera frugiperda* and other lepidopteran species. When larvae were reared on resistant plants, larval growth was reduced due to impaired nutrient utilization. Scanning electron microscopy (SEM) indicated that the peritrophic matrix (PM) was damaged when larvae fed on resistant plants or transgenic maize callus expressing Mir1-CP. To directly determine the effects of Mir1-CP on the PM in vitro, dissected PMs were treated with purified, recombinant Mir1-CP and the movement of Blue Dextran 2000 across the PM was measured. Mir1-CP completely permeabilized the PM and the time required to reach full permeability was inversely proportional to the concentration of Mir1-CP. Inclusion of E64, a specific cysteine protease inhibitor prevented the damage. The lumen side of the PM was more vulnerable to Mir1-CP attack than the epithelial side. Mir1-CP damaged the PM at pH values as high as 8.5 and more actively permeabilized the PM than equivalent concentrations of the cysteine proteases papain, bromelain and ficin. The effect of Mir1-CP on the PMs of *Helicoverpa zea*, *Danaus plexippus*, *Ostrinia nubilalis*, *Periplaneta americana* and *Tenebrio molitor* also was tested, but the greatest effect was on the *S. frugiperda* PM. These results demonstrate that the insect-inducible Mir1-CP directly damages the PM in vitro and is critical to insect defense in maize.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Corn; Cysteine protease; Plant defense; Lepidoptera; Peritrophic matrix

1. Introduction

Plants often defend themselves against attack from insect herbivores by accumulating defensive proteins. These include protease inhibitors, lectins, chitinases, oxidative enzymes and enzymes that catalyze the formation of secondary defense compounds (Constabel et al., 1999). These defensive proteins have different types of deleterious effects on insects. One of the potential targets for plant defensive proteins is the insect's peritrophic matrix (PM). The PM consists of a thin, extracellular lamina that lines the midgut of most insects (see reviews by Lehane, 1997; Barbehenn, 2001; Wang and Granados,

2001; Terra, 2001). The PM consists of a network of chitin, proteins, glycoproteins and proteoglycans (Lehane, 1997; Barbehenn, 2001; Wang and Granados, 2001; Terra, 2001). In addition to being a semi-permeable structure that is essential for nutrient passage and absorption (Wang and Granados, 2001; Terra, 2001), the PM is often the insect's first line of defense. It protects the midgut against pathogens and toxins (Barbehenn, 2001); improves digestion and protects epithelial microvilli from abrasion by food particles (Richards and Richards, 1977; Miller and Lehane, 1993; Santos and Terra, 1986; Derksen and Granados, 1988; Wang and Granados, 2001; Terra, 2001). It also provides an antioxidant defense for the midgut epithelium (Summers and Felton, 1996; Barbehenn and Stannard, 2004). Because of its importance in defense and digestion, disruption of the

---

*Corresponding author.
E-mail address: dsluthe@ra.msstate.edu (D.S. Luthe).

PM is often deleterious, if not lethal, to the insect (Wang and Granandos, 2001).

A model for the PM of lepidopteran larvae consists of chitin fibrils held together with chitin-binding proteins (CBP) (Wang and Granados, 2001; Wang et al., 2004). Disulfide bonding stabilizes the interaction of the CBPs with the chitin fibrils. Insect Intestinal Mucin (IIM), a highly glycosylated protein, associates with the CBP and protects them from degradation by digestive proteases. Agents that impair any of these interactions can disrupt PM formation and structure (Wang and Granados, 2001). For example, disruption of the chitin network with lectins or Calcofluor increased PM permeability and insect mortality (Cohen, 1987; Harper et al., 1998; Wang and Granados, 2000). The metalloprotease, enhancin, which is produced by the Trichoplusia ni granulosis virus, has been shown to specifically degrade IIM. Using a dual chamber apparatus (Spence and Kawata, 1993) designed to determine PM permeability in vitro, Peng et al. (1999) determined that in vitro treatment with enhancin increased the permeability of the T. ni PM to Blue Dextran 2000 and subsequent susceptibility to infection by the baculovirus Autographa california.

We have identified another, novel insect defense protein that appears to attack the PM (Pechan et al., 2002). A unique 33-kDa cysteine protease (Mir1-CP) rapidly accumulates in the whorls of maize (Zea mays L.) lines that have genetic resistance to leaf feeding by Spodoptera frugiperda and a number of other Lepidoptera (Davis et al., 1988). Field and laboratory experiments indicate that insects reared on these lines have reduced growth and impaired nutrient utilization (Chang et al., 2000). Larval growth also was reduced approximately 70% when larvae were reared on transgenic maize callus, over-expressing the gene encoding Mir1-CP (Pechan et al., 2000). This suggested that Mir1-CP has a direct detrimental effect on larval growth. Scanning electron microscopy (SEM) was conducted to determine if there was physical damage to the insect midgut in response to feeding on tissues that expressed Mir1-CP. SEM indicated that the PM of larvae reared on resistant maize leaves or transgenic callus was severely damaged (Pechan et al., 2002). Cracks, holes and fissures were evident in the PM and were more abundant on the luminal side of the matrix that was in contact with the food bolus. These results suggest that Mir1-CP inhibits larval growth in vivo by attacking the PM and disrupting the digestive process.

Although studies using SEM indicated that the PMs of larvae that fed on maize tissue over-expressing Mir1-CP were damaged (Pechan et al., 2002), this study was done to extend this prior work and determine if purified Mir1-CP could attack the PM in vitro. We also hoped that it would provide a quantitative estimate of the amount of permeabilization. The results reported here indicate that Mir1-CP completely permeabilizes the PM and that the time required for complete permeabilization depends on the concentration of Mir1-CP. Although it has been shown

that baculovirus metalloprotease, enhancin, attacks the PM of T. ni (Peng et al., 1999), we believe this is the first report of a plant protease damaging the insect PM.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Fisher Biotech (Fair lawn, NJ). HPLC columns (size exclusion and reverse phase chromatography) were purchased from Phenomenex (Torrance, CA). The MALDI-TOF calibration kit was purchased from Perceptive Biosystems (Foster city, CA). Commercial proteases with the following specific activities were obtained from Sigma (St. Louis, MO): papain (33 U/mg), bromelain (8.7 U/mg), ficin (19 U/mg), thermolysin (91 U/mg) and chymotrypsin (52 U/mg). Deionized water (18 MΩ) was used in all experiments.

2.1.1. Insect rearing

All 4th instar larval colonies of S. frugiperda, Helicoverpa zea, Periplaneta americana, Danaus plexippus, Ostrinia nubilalis, and Tenebrio molitor, reared on wheat germ artificial diet (Davis, 1976) with a photoperiod of 16:8 were supplied by the USDA insect rearing department located at Mississippi State University.

2.2. Purification of Mir1-CP

Mir1-CP was expressed in the hemolymph of S. frugiperda instead of Trichoplusia ni larvae as previously described (Pechan et al., 2004). Recombinant Mir1-CP was purified from the hemolymph using size exclusion and reverse phase HPLC. For size exclusion HPLC, 10 μl hemolymph was applied to a TSK-Gel column (7.8 mm ID × 30 cm dimension and 6 μm particle size, Phenomenex) and was eluted in 1 M phosphate buffer (pH 7 ± 0.2) at flow rate of 0.75 ml/min and a pressure of 33 bar using an Hewlett Packard 1050 high-pressure liquid chromatography. The column was pre-calibrated with known standards in the 11–60 kDa range. Protein elution was monitored at 214 nm using a Hitachi UV detector. Fractions (0.5 ml) corresponding to 33-kDa mass of Mir1-CP were collected between seventh and eighth minute based on the calibration. Eluted fractions with protease activity were subjected to further purification using a pre-calibrated reverse phase chromatographic column (250 × 2 mm dimension, 5 μm particle size), with gradient elution from 90% H_2O/10% acetonitrile to 70% H_2O/30% acetonitrile, 30 min; to 90% H_2O/10% acetonitrile, 5 min; 10% H_2O/90% acetonitrile, 4 min; to 90% H_2O/10% acetonitrile, 1 min, at a flow rate of 0.5 ml/min and pressure of 3129 psi. Fractions (0.5 ml) collected at 12 min with protease activity were subjected to a second round of reverse phase HPLC using a modified gradient elution of 90% H_2O/10% acetonitrile to 60% H_2O/40% acetonitrile, 30 min; to 90% H_2O/10% acetonitrile, 5 min; to 10% H_2O/90% acetonitrile, 4 min; to 90%

H$_2$O/10% acetonitrile, 1 min; while maintaining all other parameters as the previous HPLC runs. Enzymatically active fractions were concentrated by vacuum drying and stored at −80 °C.

2.3. Enzyme activity determination and immunoblotting

Protease activity in the HPLC fractions was determined using an in-gel activity assay (Michaud et al., 1993) or a rapid protease microassay (Mohan et al., 2005, in press). To determine the specific activity of the purified enzyme, protease activity was determined in a reaction mixture containing 0.4 M sodium phosphate (pH 6.0) containing 8 mM cysteine and 4 mM EDTA) and 1.25–17.5 μM of Z-Phe-Arg-AMC (Bachem, Switzerland) as substrate (Kirschke and Shaw, 1981; Tchoupe, 1991). Each reaction was incubated for 2 min at 37 °C and then terminated by adding 50 mM iodoacetic acid in acetate buffer (pH 4.3). The amount of AMC liberated from substrate was monitored spectrofluorometrically (HOEFER scientific, CA) at 460 nm. One unit of protease activity was defined as the release of 1 μmol of AMC per minute. Protein concentration was determined using the Bradford method (Bradford, 1976) with BSA as a standard. Immunoblotting of the HPLC fractions was conducted as previously described (Pechan et al., 2004).

2.4. Mass Spectrometry (MALDI-TOF MS)

The molecular mass of the purified Mir1-CP was determined using an ABI Voyager Elite MALDI-TOF MS (Matrix Activated Light Desorption Ionization-Time Of Flight Mass Spectroscopy) Voyager^{TM} Experimental station (Perspective Biosystems) (Mikes and Man, 2003). Fractions were mixed with sinapinic acid matrix in the ratio of 1:24, and excited using a nitrogen laser intensity of 3000 at 20 kV, 94% grid voltage, and 100 ns extraction delay time. Each spectrum was an average of 50 laser shots. Data Explorer^{TM} software (Perceptive Biosystems) was used for further analysis of spectra.

2.5. Permeability apparatus and measurement

The permeability apparatus was modeled after that used by Spence and Kawata (1993) and Peng et al. (1999). The main body of the permeability apparatus was machined from a solid Teflon rod (25.4 mm diameter). The apparatus consists of two cells with a mating surface of 25 mm diameter at one end of the cell. A well of 12.5 mm diameter × 21 mm depth was made in each cell 33 mm from the mating surface. The bottom of the well was connected to the mating surface via a 9.6 mm diameter channel, fitted with a hole (2 mm diameter) drilled to within 1 mm of the other end of the cylinder. The two ends of the cylinder were connected via a channel of 0.7112 mm diameter. To maintain proper alignment during assembly, the cylinder of one cell was machined such that the cylinder was recessed 1 mm into the cell, whereas the cylinder of the other cell was machined such that the cylinder protruded 1 mm out of the mating surface. These two surfaces insured proper alignment of the two cells when assembled. To further enhance the assembly of the apparatus, the two cells were keyed using a 2–56 × 25 mm screw on the mating surface of each cell. The solutions in the wells were stirred with a 1.5 mm (diameter) × 8 mm (length) micro-stir bar (Fisher Scientific, NJ). Proper mixing of the solutions was further enhanced by circulating the solution from the mating end of the cylinder back into the well using a dual channel peristaltic pump (Watson-Malow 400 F/DM2) running at 1 ml/min.

The PM was dissected from 4th instar *S. frugiperda* larvae, reared on artificial diet (Davis, 1976). The PMs of other 4th instar larval species were similarly obtained. The midgut of the intestinal tract was removed and transferred to a Petri dish containing modified Weever's saline (Weevers, 1966). The midgut was cut longitudinally and the PM containing the food bolus was removed. The PM was then cut opened longitudinally with fine scissors, rinsed in Weever's saline and aligned flat over the pore of the permeability apparatus. The orientation of PM was established in such a way that the lumen side of the PM faced the chamber that contained Mir1-CP. To insure that the PM was not damaged during dissection, a 30 min pre-run was performed by adding 2 ml of Blue Dextran 2000 (diameter: 99 nm, Pharmacia Biotech, Inc., Piscataway, NJ) solution (10 mg/ml in Weever's saline) to the lumen side of the chamber. The same volume of modified Weever's saline was simultaneously added to the epithelial-side chamber to avoid hydrostatic stress on the PM. After the pre-run, both chambers were carefully emptied. A mixture containing 10 mg of Blue Dextran 2000 in 1 ml of Weever's saline containing 0.64 mg/ml of Mir1-CP, at pH 8.5 was added to the lumen-side chamber. An equal volume of Weever's saline was added to the epithelial-side chamber simultaneously. The test was conducted for 30 min at room temperature (25 °C). Aliquots (0.1 ml) were removed from both chambers of the apparatus at 5 min intervals and refilled simultaneously. The concentration of Blue Dextran 2000 in each aliquot was determined by measuring the optical density at 610 nm. The experiment was repeated six times using a new, undamaged PM for each replicate.

3. Results

3.1. Purification of recombinant Mir1-CP from hemolymph

Recombinant Mir1-CP was purified from hemolymph using size exclusion and reverse phase HPLC. The fraction corresponding to 33 kDa, the size of Mir1-CP (Fig. 1a), was collected by size exclusion HPLC and further purified by two reverse phase chromatography steps (Fig. 1b). The single peak from the last reverse phase HPLC showed a band of protease activity of approximately 33 kDa when it was analyzed by an in-gel activity assay (Fig. 2a). It cross-
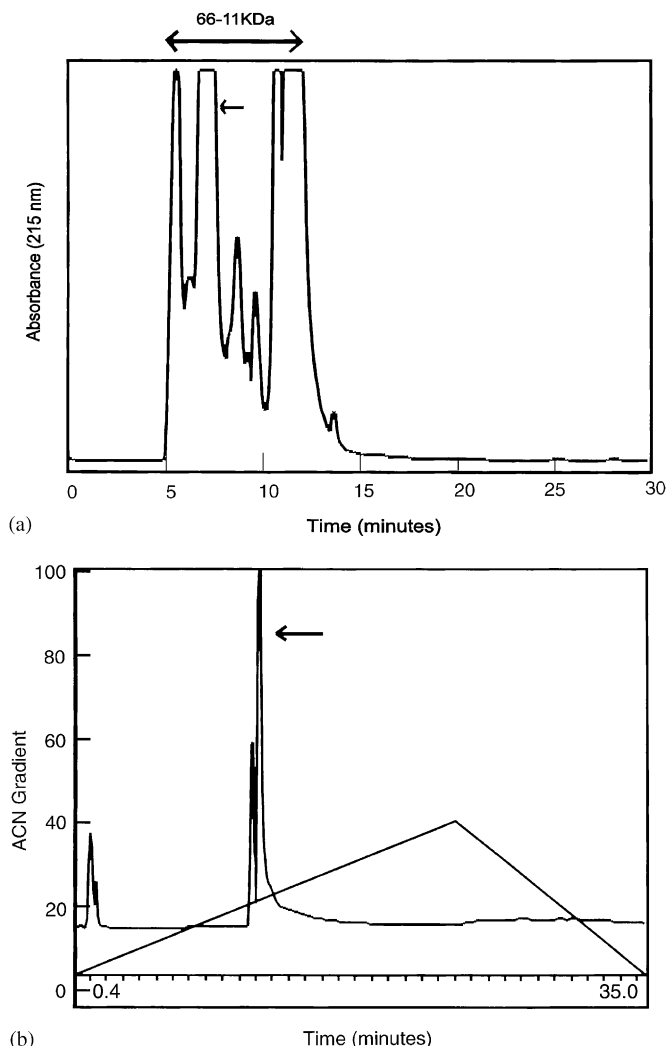
(a)

(b)

Fig. 1. Cysteine protease isolation and purification using size exclusion (a) and reverse phase (b) HPLC. Arrows mark the position of the 33 kDa peak corresponding to Mir1-CP.
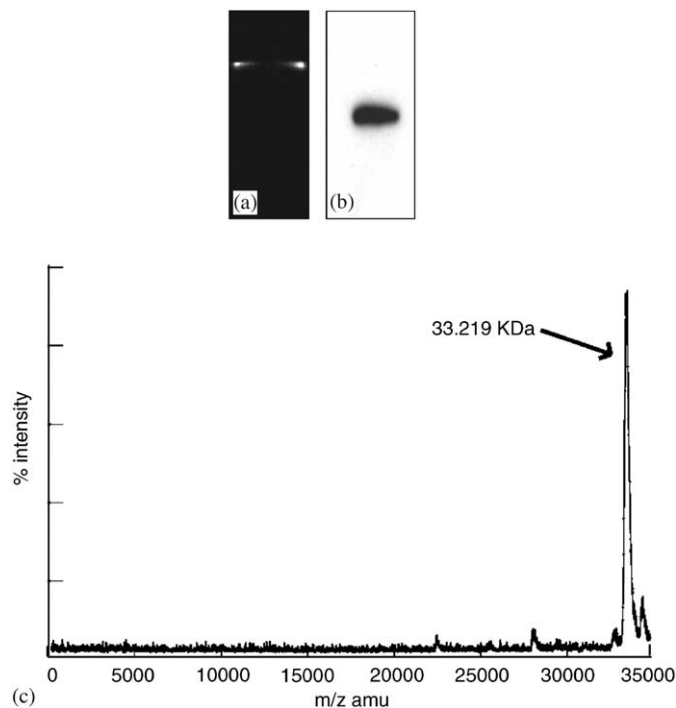

Fig. 2. Analysis of Mir1-CP purity and activity. (a) In-gel protease activity assay of the purified HPLC fraction. (b) Immunoblot analysis of the purified HPLC fraction using antibody specific to Mir1-CP. (c) MALDI-TOF MS analysis of the purified HPLC fraction.
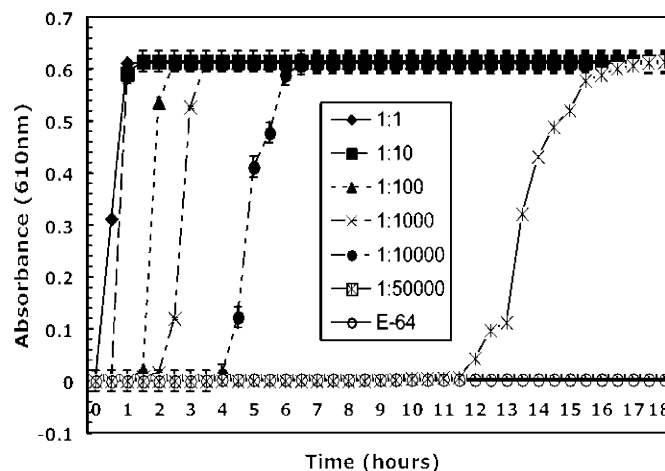

Fig. 3. *S. frugiperda* PM permeability in response to various concentrations of recombinant Mir1-CP as measured by Blue Dextran 2000 flow across the PM. (♦) Pure Mir1-CP at 0.6 mg/ml, (■) 60 μg/ml (1:10), (▲) 6 μg/ml (1:100), (×) 600 ng/ml (1:1000), (●) 60 ng/ml (1: 10000), (∗) 12 ng/ml (1:50000), (○) represents three different samples: (1) no Mir1-CP, (2) Mir1-CP (0.6 mg/ml) preincubated with 1 mg/ml E-64 treatment, (3) Mir1-CP diluted 1:60000 (10 ng/ml). PM treatments were conducted at pH 8.5 and 30 °C. The specific activity of undiluted Mir1-CP was 9 U/mg. Standard error bars are based on six different PM preparations tested.

reacted with antibody to Mir1-CP isolated from maize plants (Fig. 2b), and had a molecular mass of 33211.9 Da when analyzed by MALDI-TOF MS (Fig. 2c). Proteolytic activity was blocked when the enzyme was preincubated with the cysteine protease inhibitor E64 (data not shown). A fluorometric assay using Arg-Phe-AMC as substrate was used to determine the specific activity of the purified Mir1-CP (0.6 mg protein/ml) and it was 9 U/mg protein.

3.2. In vitro analysis of PM permeability

3.2.1. PM permeability

To determine if Mir1-CP could damage the PM in vitro, freshly dissected PMs from *S. frugiperda* were placed in the permeability apparatus and the flow of Blue Dextran between the chambers was measured. Following an initial lag phase of 5 min, PM permeability increased sharply when the lumen side was treated with the highest concentration (0.6 mg/ml or 5.4 U) of Mir1-CP. Blue

Dextran leakage across the PM ceased after 30 min and reached a steady state absorbance of 0.6 (Fig. 3). The initial Blue Dextran absorbance prior to addition of Mir1-CP was approximately 1.3, thus it appeared that the PM became

completely permeable to Blue Dextran after 30 min. When the PM was treated without Mir1-CP or with Mir1-CP pre-treated with the specific cysteine protease inhibitor E-64, there was no permeability to Blue Dextran (Fig. 3). When the epithelial side of the PM was treated with Mir1-CP, additional time was required to reach steady state permeability and complete permeability was not attained (Fig. 4).

When larvae were fed on resistant maize plants it is unlikely that they encounter concentrations of Mir1-CP as high as 0.6 mg/ml. Therefore, PMs were treated with serial dilutions of the enzyme to determine the lowest effective concentration. As the concentration decreased, the length of time required to reach steady state permeability increased. Permeability was complete after 15 h incubation at the lowest effective concentration (12 ng/ml; 1/50000 dilution). Regardless of the Mir1-CP concentration that was tested, complete permeability was attained. A 1/60000 dilution of Mir1 did not permeabilize the PM.

3.2.2. pH and temperature

To determine if Mir1-CP could attack the PM at alkaline pH values that are typically found in the lepidopteran midgut, PM permeability was measured at pH 8.5, 9 and 10. At pH 8.5 and 9.0, it took approximately 13 and 17 min to reach one-half maximal permeability, respectively, and complete permeability was attained after 25 min (Fig. 5). At pH 10.0, 22 min were needed to reach one-half maximal permeability and complete permeability was not attained.

The effect of temperature on the ability of Mir1-CP to damage the PM also was tested. The recombinant enzyme was preincubated at 30 °C for 30 min, overnight (approximately 12 h), or 24 h prior to testing in the permeability apparatus. There was little difference in the permeability attained when Mir1-CP was preincubated for 30 min or

Fig. 5. *S. frugiperda* PM permeability in response to Mir1-CP and pH as measured by Blue Dextran 2000 flow across the PM. PMs were incubated at 30 °C with Mir1-CP (0.64 mg/ml) at pH 8.5 (●), pH 9.0 (■) and pH 10 (▲). The specific activity of Mir1-CP was 9 U/mg. Standard error bars are based on six different PM preparations tested.

Fig. 6. *S. frugiperda* PM permeability in response to Mir1-CP preincubation temperature as measured by Blue Dextran 2000 flow across the PM. Prior to PM treatment at pH 8.5 and 30 °C, Mir1-CP (0.64 mg/ml) was incubated at 30 °C for 30 min (♦), 30 °C overnight (12 h) (■), 30 °C for 24 h (▲), 40 °C for 1 min (×), 40 °C for 2 min (□) and 40 °C for 3 min (●). The specific activity of Mir1-CP was 9 U/mg. Standard error bars are based on six different PM preparations tested.

overnight, (Fig. 6), but pre-incubation at 30 °C for 24 h increased the time required to reach complete permeability. When Mir1-CP was preincubated at 40 °C for 1 min, it was still active, but complete permeability was not attained. Preincubation at 40 °C for longer periods of time prevented PM damage by Mir1-CP.

3.2.3. Enzyme specific permeability

To determine if the ability to permeabilize the PM was specific to Mir1-CP, we tested the three additional plant-derived cysteine proteases at a concentration of 0.6 mg/ml, which was equivalent to the following numbers of units per treatment: bromelain, 5.2 U; papain, 19.8 U; ficin, 11.4 U. In comparison with Mir1-CP, treatment of the PM with
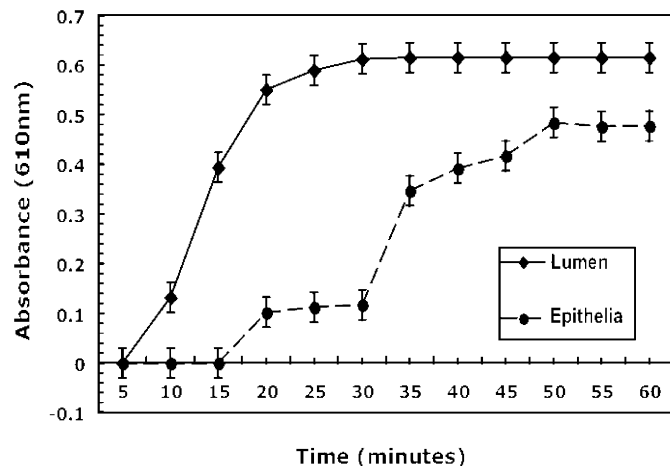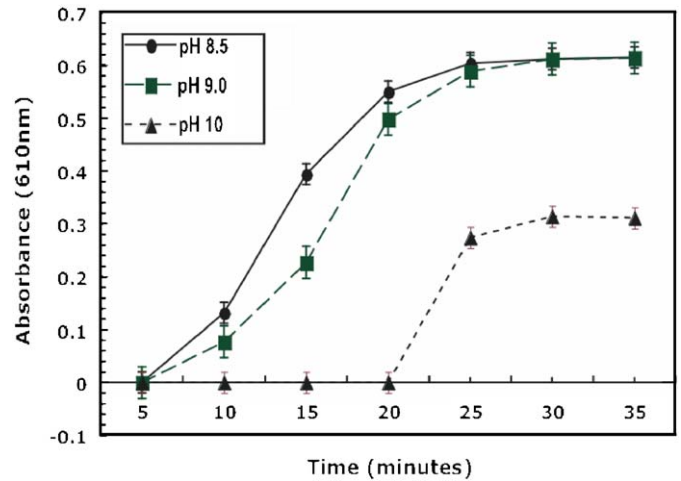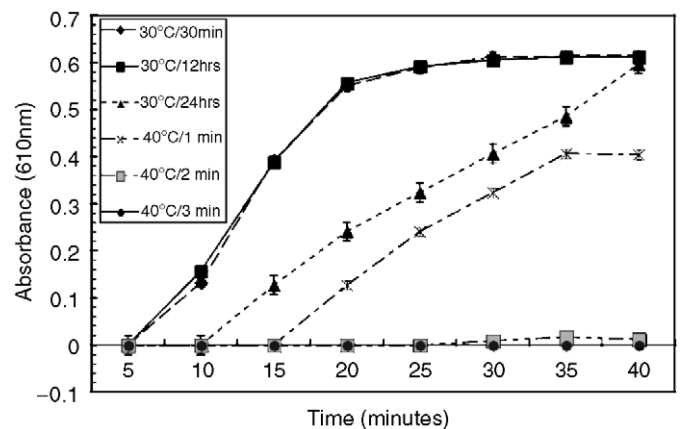

Fig. 4. *S. frugiperda* PM permeability in response to Mir1-CP and PM orientation as measured by Blue Dextran 2000 flow across the PM. The epithelial (●) and luminal (♦) sides of the PM were treated with Mir1-CP at 0.64 mg/ml at pH 8.5 and 30 °C. The specific activity of Mir1-CP was 9 U/mg. Standard error bars are based on six different PM preparations tested.
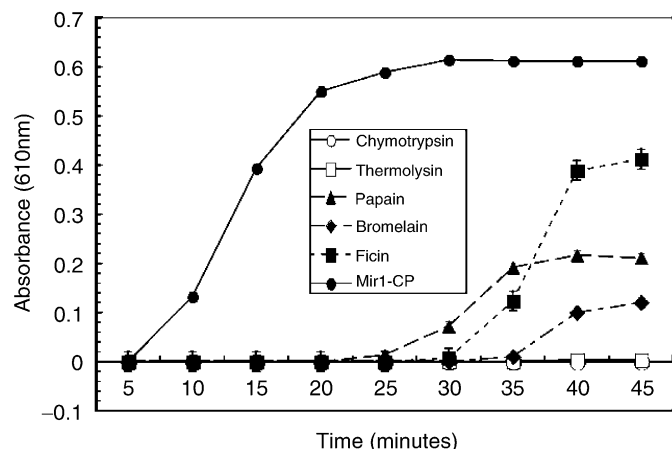
Fig. 7. *S. frugiperda* PM permeability in response to various proteolytic enzymes as measured by Blue Dextran 2000 flow across the PM. PMs were treated with 0.64 mg/ml of the cysteine proteases Mir1-CP (●), bromelain (♦), papain (▲), ficin (■) and the serine proteases, chymotrypsin (O) and thermolysin (□) at pH 8.5 at 30 °C. The specific activities of the enzymes are given in Materials and Methods. Standard error bars are based on six different PM preparations tested.
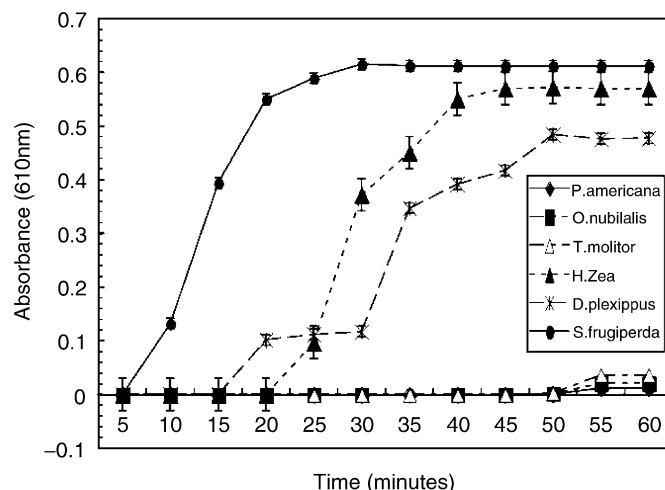

Fig. 8. Effect of Mir1-CP on the permeability of PMs isolated from several different insect species as measured by Blue Dextran 2000 flow across the PM. The PMs of *S. frugiperda* (●), *H. zea* (▲), *D. plexippus* (∗), *O. nubialis* (■), *P. americana* (♦) and *T. molitor* (△) were treated with 0.64 mg/ml of Mir1-CP at pH 8.5 and 30 °C. The specific activity of Mir1-CP was 9 U/mg. Standard error bars are based on six different PM preparations tested.

each of the cysteine proteases increased the time required to reach steady state permeability and they did not completely permeabilize the PM (Fig. 7). The steady state permeability resulting from papain, bromelain and ficin treatments was approximately 70%, 33% and 17%, respectively, of that caused by Mir1-CP. At higher pH values (pH 9 and 10) these proteases had no effect on PM permeability. The two serine proteases, chymotrypsin (31.2 U/treatment) and thermolysin (54.6 U/treatment) did not permeabilize the PM at pH 8.5 (Fig. 7), at the other pH values, or temperatures tested (data not shown).

3.2.4. Mir1-CP effect on the PMs from other insect species

To determine if Mir1-CP effect was specific to the *S. frugiperda* PM, we tested its effect on three other Lepidopterans, *H. zea*, *D. plexippus* and *O. nubilalis* (Fig. 8). Although Mir1-CP was most effective against *S. frugiperda*, it also permeabilized the PMs of *H. zea* and *D. plexippus* when they were incubated with 0.6 mg/ml of Mir1-CP at pH 8.5. More time was required to reach one-half permeability and complete permeability was not attained for either insect. However, Mir1-CP appeared to be more effective in permeabilizing the PM of *H. zea* than *D. plexippus*. Mir1-CP did not affect the permeability of the *O. nubilialis* PM. We also tested the effect of Mir1-CP on PMs isolated from the Orthropteran *P. americana* and the Coleopteran *T. molitor*. Mir1-CP did not increase the permeability of these PMs under any of the conditions tested (Fig. 8).

4. Discussion

In previous work (Pechan et al., 2002), we used SEM to show that PMs of *S. frugiperda* larvae were severely

damaged when they fed on transgenic plant material over-expressing Mir1-CP. Although there was little PM damage when larvae fed on controls, there still was the possibility that damage could be due to the interaction of Mir1-CP with other plant components. This study was conducted to determine if purified Mir1-CP could permeabilize the PM in vitro. This should provide unequivocal evidence that Mir1-CP caused PM damage. The in vitro system also allowed us to test the effects of pH and temperature on Mir1-CP activity.

The study was facilitated by our ability to express a large quantity of Mir1-CP using a modified baculovirus system (Pechan et al., 2004). Mir1-CP was expressed in *S. frugiperda* larvae and the recombinant enzyme was purified from the hemolymph. Typically, we obtained approximately 0.6 mg of purified Mir1-CP for each 10 μl of hemolymph. Considering that one larva contains between 100 and 200 μl of hemolymph, it is possible to obtain a significant amount of Mir1-CP from a few larvae. Characterization of recombinant Mir1-CP indicted that it had the same properties as the plant-derived enzyme.

When PMs isolated from *S. frugiperda* were incubated with enzymatically active recombinant Mir1-CP, they became completely permeable to Blue Dextran 2000. Complete permeability to Blue Dextran was obtained at every Mir1-CP concentration tested, but the time required to reach complete permeability increased as the concentration decreased. The lowest effective concentration was a 1/50000 dilution of stock Mir1-CP or 1.08×10^{-4} U. This was equivalent to 12 ng/ml, which is likely to be a physiologically relevant concentration in the maize whorl. To determine if proteolytic activity was required to permeabilize the PM, we blocked cysteine protease activity

by pre-treating Mir1-CP with the specific inhibitor E64. In the presence of the inhibitor, Blue Dextran movement through the PM was abolished, which demonstrated that cysteine protease activity is required to permeabilize the PM.

According to Dow (1992) the gut pH of *S. frugiperda* is alkaline with pH range between 8.5 and 9. However, the pH optimum for Mir1-CP is acidic (Jiang et al., 1995). For Mir1-CP to function in vivo, it must retain some enzymatic activity under alkaline conditions corresponding to those found in the gut. The in vitro experiments conducted in this study indicated that Mir1-CP could permeabilize the PM in vitro under alkaline conditions up to pH 10. Consequently, it is likely that Mir1-CP has similar activity in vivo.

Heat treatment of Mir1-CP was conducted to determine the effect of temperature on activity. The temperatures of 30 and 40 °C were selected because they are at the lower and upper range of temperatures that might be encountered by the host plant and insect in the field. Mir1-CP was still fully active after incubation at 30 °C for 12 h and retained some activity after a 24 h incubation. Preincubation at 40 °C for longer than 1 min probably inactivated the enzyme. Because preincubation at 40 °C abolished activity, higher temperatures were not tested.

Recent studies by Konno et al. (2004) indicated that cysteine proteases in the latex of papaya (*Carica papaya*) and fig (*Ficus virgata*) are crucial in defending plants from feeding by *Samia ricini*, *Mamestra brassicae* and *Spodoptera litura*. Therefore, the efficacy of three additional plant-derived cysteine proteases was tested. Papain, bromelain and ficin only partially permeabilized the *S. frugiperda* PM, even when they were used at higher concentrations than Mir1-CP. The two serine proteases, chymotrypsin and thermolysin, had no effect on PM permeability. The effect of Mir1-CP on the PM permeability was more pronounced when the luminal side was exposed to the enzyme. When the epithelial side was exposed to Mir1-CP, it took longer to reach steady state permeability and it was approximately 20% less than completely permeable. These data suggested that the protein or proteins attacked by Mir1-CP are more accessible on the luminal side of the PM than on the epithelial side.

Mir1-CP is found in maize germplasm that is resistant to a number of stem-boring Lepidoptera (Davis et al., 1988). Consequently, we wanted to determine if it could attack the PMs of other insects. Mir1-CP effectively permeabilized the PM of the two noctuids tested, *H. zea* and *S. frugiperda*. It affected the PM of the danaid, *D. plexippus*, but not that of the crambid, *O. nubilalis*. This implies that there may be evolutionary differences in PM structure and sensitivity to Mir1-CP among the Lepidoptera. It is interesting that the insect-resistant maize germplasm inhibits the growth of a number of Lepidoptera including noctuids, crambids and pyralids in the field and bioassays. Because insect resistance in these maize lines is a multigene trait, it is likely that additional factors are needed to confer resistance to a wide range of

lepidopterans. The PMs of *P. americana* and *T. molitor* also were resistant to Mir1-CP-induced permeability. Evolutionary differences among species in PM formation or structure (Barbehenn and Martin, 1995) could greatly affect the interaction of Mir1-CP with the PM chitin and proteinaceous components.

We have demonstrated that a unique maize cysteine protease Mir1-CP is capable of directly permeabilizing the PM of several lepidopteran larvae. Inhibition of Mir1-CP activity with E64 prevented permeabilization indicating that proteolytic activity is required to damage the PM. It is likely that Mir1-CP attacks and degrades PM proteins, but we currently do not know if it attacks a specific protein like IIM, or if it non-specifically degrades all PM proteins. Nevertheless, it appears that Mir1-CP plays a critical role in maize defense against herbivory by lepidopteran larvae.

Acknowledgments

References

Barbehenn, R.V., 2001. Roles of peritrophic membranes in protecting herbivorous insects from ingested plant allelochemicals. Archives of Insect Biochemistry and Physiology 47, 86–99.

Barbehenn, R.V., Martin, M.M., 1995. Peritrophic envelope permeability in herbivorous insects. Journal of Insect Physiology 41, 303–311.

Barbehenn, R.V., Stannard, J., 2004. Antioxidant defense of the midgut epithelium by the peritrophic envelop in caterpillars. Journal of Insect Physiology 50, 783–790.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry 72, 248–254.

Chang, Y.M., Luthe, D.S., Davis, F.M., Williams, W.P., 2000. Influence of whorl region from resistance and susceptible corn genotypes of fall armyworm (Lepidoptera: Noctuidae) growth and development. Journal of Economic Entomology 93, 478–483.

Cohen, E., 1987. Chitin biochemistry: synthesis and inhibition. Annual Review of Entomology 32, 71–93.

Constabel, C., Bergey, D.R., Ryan, C.A., 1999. A survey of herbivore-inducible proteins and phytochemicals. In: Agrawal, A.A., Tuzun, S., Bent, E. (Eds.), Induced Plant Defenses Against Pathogens and Herbivores. American Phytopathological Society, St. Paul, MN, pp. 137–166.

Davis, F.M., 1976. Rearing the southwestern corn borer and fall armyworms at Mississippi State. Technical Bulletin—Mississippi Agricultural and Forestry Experiment Station 75, pp. 54–67.

Davis, F.M., Williams, W.P., Mihm, J.A., Barry, B.D., Overman, L.J., Wiseman, B.R., Riley, T.J., 1988. Resistance to multiple lepidopterous species in tropical derived corn germplasm. Technical Bulletin—Mississippi Agricultural and Forestry Experiment Station 157, pp. 1–6.

Derksen, A.C., Granados, R.R., 1988. Alteration of a lepidopteran peritrophic membrane by baculoviruses and enhancement of viral infectivity. Virology 78, 3091–3100.

Dow, J.A., 1992. pH gradients in lepidopteran midgut. Journal of Experimental Biology 172, 355–375.

Harper, M.S., Hopkins, T.L., Czapla, T.H., 1998. Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in European corn borer (*Ostrinia nubilalis*) larvae. Tissue Cell 30, 166–176.

Jiang, B.H., Siregar, U., Willeford, K.O., Luthe, D.S., Williams, W.P., 1995. Association of a 33 KD cysteine protease found in maize callus with the inhibition of fall armyworm larval growth. Plant Physiology 108, 1631–1640.

Kirschke, H., Shaw, E., 1981. Rapid interaction of cathepsin L by Z-Phe-PheCHN12 and Z-Phe-AlaCHN2. Biochemical and Biophysical Research Communications 101, 454–458.

Konno, K., Hirayama, C., Nakamura, M., Tateishi, K., Tamura, Y., Hattori, M., Kohno, K., 2004. Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. The Plant Journal 37, 370–378.

Lehane, M.J., 1997. Peritrophic matrix structure and function. Annual Review of Entomology 42, 525–550.

Michaud, D., Faye, L., Yelle, S., 1993. Electrophoretic analysis of plant cysteine and serine proteinases using gelatin containing polyacrylamide gels and class specific protease inhibitors. Electrophoresis 14, 94–98.

Mikes, L., Man, P., 2003. Purification and characterization of saccharide-binding protein from penetration glands of *Diplostromum pseudos-pathaceum*—a bifunctional molecule with CP activity. Parasitology 127, 69–77.

Miller, N., Lehane, M.J., 1993. Ionic environment and the permeability properties of the peritrophic membrane of *Glossina moristans moristans*. Journal of Insect Physiology 39, 139–144.

Mohan, M., Ma, P.W.K., Luthe, D.S., 2005. Rapid qualitative protease microassay (RPM). Journal of Biochemical and Biophysical Methods 64, 182–188.

Pechan, T., Ye, L., Chang, Y.M., Mitra, A., Lin, L., Davis, F.M., Williams, W.P., Luthe, D.S., 2000. A unique 33 KD cysteine proteinase accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other Lepidoptera. Plant Cell 12, 1031–1040.

Pechan, T., Cohen, A., Williams, W.P., Luthe, D.S., 2002. Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proceedings of the National Academy of Science USA 99, 13,319–13,323.

Pechan, T., Ma, P.W.K., Luthe, D.S., 2004. Heterologous expression of maize (*Z. mays.* L) Mir1 cysteine proteinase in eukaryotic and prokaryotic expression systems. Protein Expression and Purification 34, 134–141.

Peng, J., Zhong, J., Granados, R.R., 1999. A baculovirus enhancin alters the permeability of a mucosal midgut peritrophic matrix from lepidopteran larvae. Journal of Insect Physiology 45, 159–166.

Richards, A.G., Richards, P.A., 1977. The peritrophic membrane of insects. Annual Review of Entomology 22, 219–240.

Santos, C.D., Terra, W.R., 1986. Distribution and characterization of oligomeric digestive enzymes from *Erinnyis ella* larvae and inferences concerning secretory mechanisms and the permeability of the peritrophic membrane. Insect Biochemistry 16, 123–321.

Spence, K.D., Kawata, M.Y., 1993. Permeability characteristics of the peritrophic membranes of *Manduca sexta*. Journal of Insect Physiology 39, 785–790.

Summers, C.B., Felton, G.W., 1996. Peritrophic envelope as a functional antioxidant. Archives of Insect Biochemistry and Physiology 32, 131–142.

Tchoupe, J.R., 1991. Photometric or fluorometric assay of cathepsin B, L and H and papain using substrates with an aminotrifluoromethylcoumarin leaving group. Biochemica et Biophysica Acta 1076, 149–151.

Terra, W.R., 2001. The origin and functions of the insect peritrohic membrane and peritrophic gel. Archives of Insect Biochemistry and Physiology 47, 47–51.

Wang, P., Granados, R.R., 2000. Calcofluor disrupts the midgut defense system in insects. Insect Biochemistry and Molecular Biology 30, 135–143.

Wang, P., Granados, R.R., 2001. Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. Archives of Insect Biochemistry and Physiology 47, 110–118.

Wang, P., Li, G., Granados, R.R., 2004. Identification of two new peritrophic membrane proteins from larval *Trichoplusia ni*: structural characteristics and their functions in the protease rich insect gut. Insect Biochemistry and Molecular Biology 34, 217–227.

Weevers, R.D., 1966. A lepidopteran saline: effects of inorganic cation concentrations on sensory, reflex and motor responses in a herbivorous insect. Journal of Experimental Biology 44, 163–175.